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ARTICLE

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The charge state of an ion channel controls neutral polymer entry into its pore

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Abstract Electrostatic potentials created by fixed or induced charges regulate many cellular phenomena including the rate of ion transport through proteinaceous ion channels. Nanometer-scale pores of these channels also play a critical role in the transport of charged and neutral macromolecules. We demonstrate here that, surprisingly, changing the charge state of a channel markedly alters the ability of *nonelectrolyte* polymers to enter the channel's pore. Specifically, we show that the partitioning of differently-sized linear nonelectrolyte polymers of ethylene glycol into the *Staphylococcus aureus* α-hemolysin channel is altered by the solution pH. Protonating some of the channel side chains decreases the characteristic polymer size (molecular weight) that can enter the pore by ~25% but increases the ionic current by ~15%. Thus, the "steric" and "electric" size of the channel change in opposite directions. The results suggest that effects due to polymer and channel hydration are crucial for polymer transport through such pores.

Key words Ion channel · Transport · Polymers · Hydration · Water structure · Alpha hemolysin · Poly(ethylene glycol)

Introduction

Electrostatic potentials at biological interfaces play an important role in a wide variety of cellular processes (McLaughlin 1989). These potentials, which arise from charges on or near the cell surface, alter the concentration of mobile ions in the aqueous phases directly adjacent to the membrane. Thus, the surface concentration of ions like Ca^{2+}, which are involved in cell signaling and second messenger reactions, depend markedly on the membrane surface potential.

The membrane surface charge also affects the rate of ion transport through channels (Green and Andersen 1991). For example, the conductance of the gramicidin A channel (Apell et al. 1979), potassium channels from sarcoplasmic reticulum (Bell and Miller 1984), calcium-activated potassium channels (Moczydlowski et al. 1985), L-type calcium channels (Coronado and Affolter 1986), and the channel formed by *Staphylococcus aureus* α-hemolysin (Krasilnikov and Sabirov 1989) are affected by the charge density of lipids in planar bilayer membranes in which those channels were reconstituted. For cation selective channels, negatively charged lipids increased the single-channel conductance, especially at low concentrations of the permeant species. Channel conductance can also be modified by adding fixed charges, via chemical modification, to the channel entrance (Apell et al. 1977).

Recent experimental evidence demonstrated that fixed charges on the channels themselves play an important role in ion selectivity in both relatively narrow Ca^{2+} pores (Yang et al. 1993) and wider pores like the voltage dependent anion channel (Blachly-Dyson et al. 1990). Changing the charge state of ionizable residues in an ion channel also changes the conductance of several different channels (Prod'hom et al. 1987; Bezrukov and Kasianowicz 1993; Root and MacKinnon 1994; Kasianowicz and Bezrukov 1995).

We demonstrate here that the charge state of an ion channel also affects the ability of nonelectrolyte polymers to enter a channel's pore. Titrating side chains on the channel formed by *Staphylococcus aureus* α-hemolysin causes the ionic current to increase and the characteristic "cut-off" polymer size to decrease. These results illustrate the importance of electrostatic effects, probably related to the sensitivity of ion channel hydration to its charge state, for the transport of molecules regardless of their charge.

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Methods

We determined the ability of differently-sized polymers to partition into a single channel that spans a lipid bilayer membrane. Specifically, we measured the ionic current that flows through the channel's pore at a constant applied potential, V = 100 mV, in the presence of differently-sized poly(ethylene glycols), PEGs. Single channels were formed by adding less than 1 μg of α-hemolysin, also known as *S. aureus* α-toxin, to one side of a planar lipid bilayer membrane which was bathed by symmetric aqueous solutions containing 1 M NaCl, 2.5 mM MES (or HEPES) at pH 7.5 or pH 4.5 and 15% (w/w) of a given molecular weight PEG added to the salt solution. We used PEGs with molecular weights between 200 and 17000 Da (Aldrich, Milwaukee, WI and Fluka, Buchs, Switzerland). Solvent-free membranes were formed from diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Inc., Alabaster, AL) in high purity pentane (Burdick and Jackson Muskegon, MI) and the current was measured and analyzed as described earlier (Kasianowicz and Bezrukov 1995; Bezrukov, et al. 1996). The temperature was T = (24.0±1.5) °C.

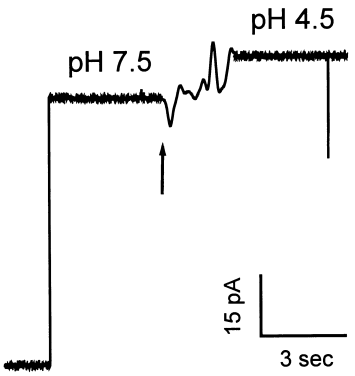

Fig. 1 Decreasing the pH causes an increase in the S. aureus α-hemolysin single channel current. The experimental recording above shows that changing the pH from 7.5 to 4.5 increases the current through the same single channel by ~15%. The initial jump in current corresponds to the formation of a single channel. The solutions bathing each side of the membrane initially contained 1.6 ml of 1 M NaCl, 5 mM citric acid, and 1 mM MOPS at pH 7.5. The solutions on both sides of the membrane were titrated to pH 4.5 by adding 5.6 μl of 2.7 N HCl to each chamber (arrow). The data was recorded at a rate of 250 points/sec and filtered at f_c = 100 Hz, except while stirring during HCl addition when it was filtered at f_c = 2 Hz. The change in current is related to the protonation of channel residues (Bezrukov and Kasianowicz 1993; Kasianowicz and Bezrukov 1995)

Results

The ionic current through the α-hemolysin channel is pH dependent. Figure 1 demonstrates that as the pH decreases from 7.5 to 4.5, the channel conductance increases by ~15%. In previous studies, we determined the binding constant of the amino acid side chains that regulate this channel's conductance and the rate constants for the association and dissociation of protons to these sites (Bezrukov and Kasianowicz 1993; Kasianowicz and Bezrukov 1995). Channels are often "sized" using conductance measurements. Is the pH-induced increase in this channel's conductance caused by a concomitant increase in the pore's diameter?

To address that question, we determined the ability of differently-sized non-electrolyte polymers of PEG, poly(ethylene glycol), to partition into the α-hemolysin channel at these two pH values. Previous studies showed that polymers smaller than a characteristic size, roughly equal to the pore diameter, partition into the α-hemolysin pore and reduce the channel's mean conductance (Krasilnikov et al. 1992; Korchev et al. 1995; Bezrukov et al. 1996).

Similar conclusions about the pore size can be drawn from polymer-induced conductance fluctuations (Bezrukov et al. 1994), which, for the α-hemolysin pore, show a striking non-monotonic dependence on the polymer molecular weight (Bezrukov et al. 1996). This effect is shown in Fig. 2, which contains a series of single channel current recordings measured in 1 M NaCl solutions at pH 4.5 or 7.5 containing either no polymer or PEG with the indicated molecular weights. In the absence of polymer, the current is relatively noise-free. Adding polymer to the aqueous

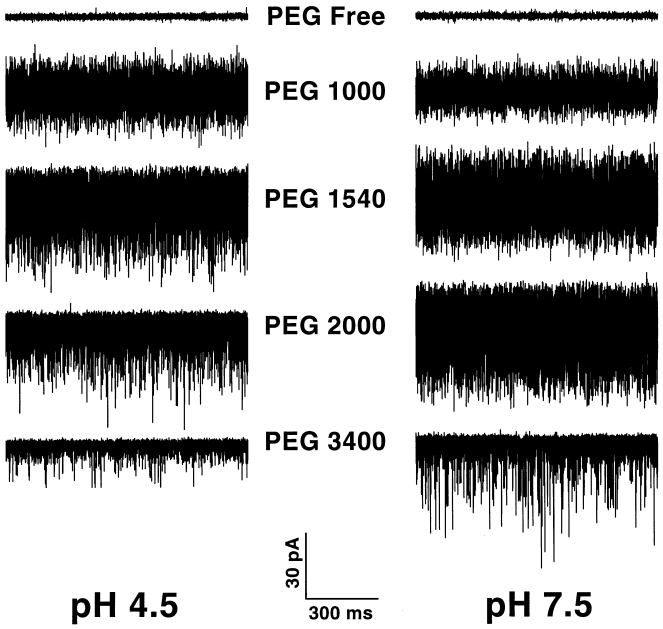

Fig. 2 Poly(ethylene glycol) causes current fluctuations that depend on both the polymer molecular weight and the solution pH. Single channel current traces at pH 4.5 and 7.5 show that the polymer-induced current noise varies non-monotonically with the PEG molecular weight at both pH values. However, note that larger polymer (PEG 2000) is required to cause the maximum current noise at pH 7.5 compared to that at pH 4.5 (PEG 1540) and that the noise caused by PEG 3400 partitioning into the pore is much larger at pH 7.5 compared to that at pH 4.5

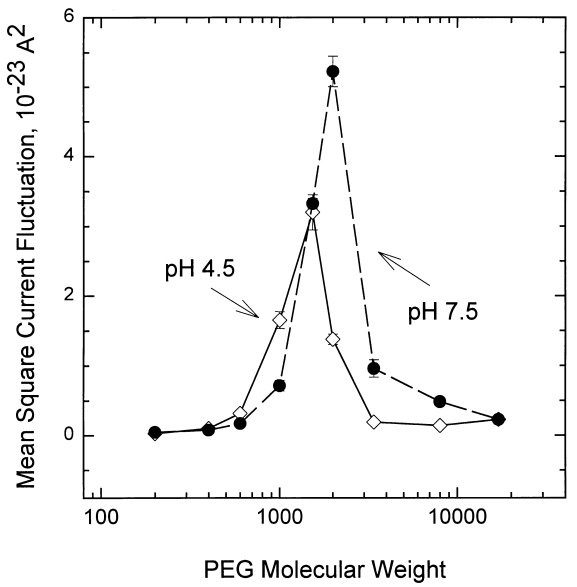

Fig. 3 The change in pH shifts the maximum of the polymer-induced current fluctuations (at 1 kHz bandwidth). Note that the polymer weight corresponding to the maximum current noise is smaller at pH 4.5 than it is at pH 7.5 (see Fig. 2). These results suggest that pore's size is larger at the higher pH value. However, from the magnitude of the single channel current, the opposite appears true (see Fig. 1). We argue that the difference in the polymer-induced noise at the two pH values is not related to a change in the physical size of the channel protein itself. Rather, it is caused by a change in the hydration of ionizable residues inside the pore

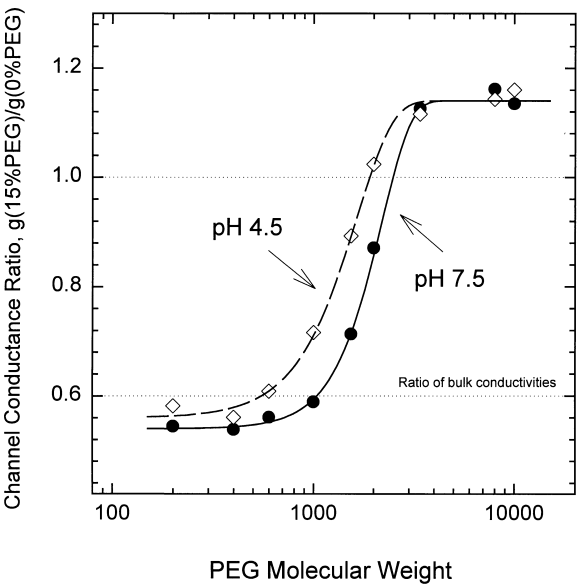

Fig. 4 Relative change in the single channel mean conductance as a function of the PEG molecular weight and pH. At pH 4.5 or 7.5, the mean conductance decreases as the PEG size decreases because smaller polymers partition into the pore. The apparent "cut-off" size of PEG entry into the pore is greater at pH 7.5 than it is at pH 4.5, which was also apparent in Figs. 2 and 3. The lines through the points for each pH value represent the least square fits of Eqs. (1) and (2) to the conductance ratio

phase causes current fluctuations that first increase and then decrease with increasing PEG molecular weight. Note, however, that the maximum current noise occurs at a characteristic molecular weight that depends on pH. The maximum current noise occurs at PEG 1540 and 2000 for pH 4.5 and pH 7.5, respectively. The difference in current noise is most visible in solutions containing PEG 3400.

Figure 3 illustrates this point quantitatively over a wider range of polymer molecular weight. The variance of the bandwidth-limited current noise, which is proportional to the low frequency current spectral density, is relatively small for short-length PEGs or long polymers that do not partition into the pore. At intermediate polymer molecular weights, the variance increases non-monotonically to a sharp peak for both pH values. As is evident from the current recordings in Fig. 2, the maximum variance occurs at a larger polymer size for the higher pH solution. Thus, although the ionic current increases as the pH is decreased (Fig. 1), the "cut-off" size describing PEG penetration into the pore *decreases* (Figs. 2 and 3).

Steady-state measurements of the pore conductance in the presence of differently sized PEGs provide further evidence that the pore's apparent size is larger at higher pH. Polymers that partition into the pore decrease the conductance. Figure 4 shows the relative change in the conductance, as a function of PEG molecular weight, compared to the conductance in the absence of polymer. High molecular weight polymers, which do not partition into the pore, increase the conductance because PEG binds water, thus increasing the electrolyte activity (Bezrukov and Vodyanoy 1993). Intermediately-sized PEGs cause the conductance to decrease with decreasing polymer molecular weight. Note that the partitioning of PEG into the pore is shifted to higher molecular weight polymer for pH 7.5 compared to that for pH 4.5.

The lines through the data are the least squares fit of a simple expression for the relative change in conductance, $g(w)/g_0$, in terms of the partition coefficient, $p(w)$ [(Eq. (2)], and the bulk conductivities in the presence and absence of polymer σ', σ (Bezrukov et al. 1996)

$$g(w)/g_0 = \beta(1-(1-\sigma'/\beta\sigma)p(w)) \qquad (1)$$

where w is the polymer molecular weight, and $g(w)$, g_0 are the pore conductances in the presence and absence of polymer. The parameter $\beta = 1.14$ describes the increase in salt activity caused by PEG and is determined independently from PEG's water binding properties (Bezrukov and Vodyanoy 1993). The partition coefficient, which is the ratio of the average monomer density inside the pore to that in the solution outside the pore, is assumed to be of the form

$$p(w) = \mathrm{e}^{-(w/w_0)^{\alpha}} \qquad (2)$$

where α and w_0 are adjustable fit parameters (Bezrukov et al. 1996). The parameter α describes the sharpness of the transition and w_0 is the characteristic "cut-off" polymer molecular weight. For pH 4.5 $\alpha = 2.4 \pm 0.2$ and $w_0 = 1650 \pm 50$. For pH 7.5 $\alpha = 3.1 \pm 0.2$ and $w_0 = 2200 \pm 100$. Once again, the sizes of the pore deduced from ionic current measurements in polymer free solutions (Fig. 1) and

polymer partitioning (Fig. 4) change in opposite directions.

It should be noted that the hydration of PEG in the bulk is independent of pH. According to our measurements, the Na^+ activity in the presence of 15% PEG (w/w), determined with a Na^+ selective electrode, is virtually the same at pH 4.5 and 7.5 (it differs by less than 0.2%). Similarly, the bulk conductivity decrease, caused by the same concentration of polymer, is also unchanged within 0.3%. The absence of pH sensitivity of these physical properties suggests that all other properties of the polymer (e.g. radius of gyration) are also independent of pH. Thus, the observed shift in the polymer partitioning (Figs. 2, 3 and 4) is related to the changes in the hydration state of the pore's lumen (see below).

Discussion

Although ion channels are most well known for their ability to regulate the transport of ions (Hille 1992), they also control the flux of small solutes (e.g. Rostovtseva and Colombini 1996), proteins (Simon and Blobel 1991; Bustamante et al. 1995), and can allow the passage of water soluble neutral polymers (Bezrukov et al. 1994; Bezrukov et al. 1996) or even single stranded RNA and DNA (Kasianowicz et al. 1996). Clearly, these functions are intimately connected with channel structure and it is important to understand them in terms of a coherent model.

Channel sizes determined from the flux of solutes through the pore and the channel conductance correlate poorly (Finkelstein 1985). Typically, the channel pore diameter is deduced by measuring the permeability of the channel to (or partitioning of) differently-sized nonelectrolytes, as is shown above (Fig. 4). Nonelectrolyte polymers smaller than a characteristic cut-off size penetrate into the pore and lower the conductance. In the absence of specific interactions between the polymer and the channel, polymers that are too large to partition into the pore minimally affect the channel's conductance (Krasilnikov et al. 1992; Bezrukov and Vodyanoy 1993; Korchev et al. 1995; Bezrukov et al. 1996).

Channel "size" is often calculated from the conductance by assuming that the concentration and mobility of ions inside the pore are identical to those in bulk solution. However, the channel conductance, and thus the apparent electric size of a pore, is generally dependent on the charges on (Apell et al. 1977; Prod'hom et al. 1987; Bezrukov and Kasianowicz 1993; Root and MacKinnon 1994; Kasianowicz and Bezrukov 1995) or near the channel (Apell et al. 1979; Green and Andersen 1991; Bell and Miller 1984; Moczydlowski et al. 1985; Coronado and Affolter 1986; Krasilnikov and Sabirov 1989). Thus, ionic flux through channels is also controlled by physical causes other than simple geometry. Simply put, electrostatics rules in structures whose characteristic size is on the order of the Debye length. Ongoing theoretical studies underscore this point and provide rich models that can ultimately be tested (Levitt 1985; Jordon 1987; Jordon et al. 1989; Green and Andersen 1991; Barcilon 1992; Barcilon et al. 1992; Chen and Eisenberg 1993; Eisenberg 1996).

Table 1 Free energy change of a single polymer partitioning caused by channel side chain protonation

PEG weight	600	1000	1540	2000	3400
Energy difference (in units of kT)	0.07	0.21	0.51	0.83	2.00

We show here that changing the charge state of an ion channel can also dramatically alter the entry of nonelectrolyte polymers into the pore. The extent to which polymers partition into pores is obtained experimentally from the polymer-induced reduction of the channel conductance (Fig. 4, Eqs. 1 and 2) and can be used to estimate the free energy of polymer entrapment into the pore (Bezrukov et al. 1996).

The difference in free energies of a single polymer partitioning into a pore from bulk solutions at the two different pH values used in this study can be shown to be:

$$\Delta E(w)_{4.5\rightarrow 7.5} = kT \ln(p(w)_{4.5}/p(w)_{7.5}), \quad (3)$$

where k is the Boltzmann constant, T is absolute temperature, and $p(w)_{4.5}$ and $p(w)_{7.5}$ are the equilibrium polymer partition coefficients at pH 4.5 and pH 7.5, respectively. Table 1 shows estimates of the free energy change for several differently sized PEGs obtained from the data in Fig. 4. For large polymers, the change in free energy per single polymer molecule is substantial and exceeds one kT. For PEGs in the molecular weight range 600–3400, the energy difference scales with the square of the polymer length (molecular weight).

How could changing the channel's charge state alter the ability of nonelectrolyte polymers to partition into the pore? There are several possibilities. First, the binding of a charge to an amino acid side chain inside the pore will alter the properties of water therein (Green and Lu 1995; Lynden-Bell and Rasaiah 1996; Sansom et al. 1996). The protonation of carboxyl acid side chains of amino acids changes these groups from H-bond acceptors to H-bond donors. It was also shown that the hydration of charged and neutral residues is different (e.g. see computer simulations of a single "chloride" (Perera and Berkowitz 1992; Dang and Smith 1993)). The strong electric field produced by a localized charge alters the dielectric constant of the water in the pore which leads to a difference in polymer-water interactions (as compared to those in the bulk). In a simplified illustration of these effects (Fig. 5), water is more tightly bound to the protonated site, and thus unavailable to hydrate polymers inside the pore. This would cause a shift in the polymer's partitioning into the pore. Our results suggest that changing the charge state of even a few residues leads to a significant difference in the extent to which polymers partition into the pore.

Second, if we consider the interactions from the polymer's standpoint, then polymer entry into the pore will change the dielectric constant of the solution within. Amongst other

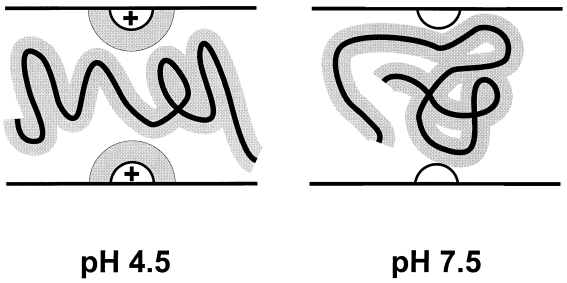

Fig. 5 Speculative model for the entropic interaction of poly(ethylene glycol) with the *S. aureus* α-hemolysin channel. The protonation of hydrophobic residues inside the pore changes their state of hydration making some of the water unavailable for polymer hydration. Thus the size of the pore, as seen by the polymer, appears smaller while the physical size of the channel protein itself is unchanged

things, this would certainly change the energetics of charge screening. In particular, strong fields caused by localized charges attract matter with greater dielectric constants. Because the dielectric constant of the polymer used in this study, PEG, is significantly lower than that of water (Feakins and French 1957), polymer is excluded at the expense of water.

It is generally believed, in the case of biological surfaces and macromolecules, that "geometrical considerations suggest that electrostatics is more important for charged planes (membranes) than for cylinders (DNA) or spheres (proteins)" (McLaughlin 1989). While that is true for macromolecules in the bulk aqueous phase, electrostatics has a significant effect on ion transport through cylindrical structures whose length scales are comparable to the Debye length. We demonstrate here that, surprisingly, electrostatics also affects the ability of nonelectrolyte polymers to partition into a cylindrical channel.

Similarly, while no one would argue that the electrostatic properties of a bilayer membrane would alter the binding of charged species to membrane-bound structures, it's conceivable that surface charge might also modify and regulate the binding and reactivity of neutral or nearly neutral solutes onto surfaces as well. In conclusion, the results of our study suggest that the hydration state of biopolymers (Parsegian et al. 1986; Timasheff 1993; Parsegian et al. 1995), which can be altered by changes in the electrostatics of their surroundings, plays an important role in transmembrane transport and cellular regulation.

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